NITROGEN FIXATION IN SYMBIOTIC MARINE SPONGES: ECOLOGICAL SIGNIFICANCE AND DIFFICULTIES IN DETECTION

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There has been considerable speculation, and some evidence, that coral reef sponges can fix atmospheric nitrogen through some of their microbial symbionts, particularly symbiotic cyanobacteria. Many Indo-Pacific coral reef sponges can satisfy much of their requirement for carbon energy compounds via translocation from photosynthetic symbionts, and a similar mechanism has been invoked to explain how some sponges could supplement the low amount of available nitrogen in clear tropical waters. Attempts to measure nitrogen fixation using the acetylene reduction test have proven technically difficult and given ambiguous results. However, fixation was demonstrated unambiguously with incorporation of the stable isotope $^{15}N_2$ into the amino acids glutamine, glutamate and aspartate of *Callyspongia muricina*, although at relatively low rates. The variability in measuring acetylene reduction in 23 sponge species is attributed to several factors: the number of cellular and matrix barriers that must be passed by acetylene and ethylene; the difficulty of maintaining sponges alive under experimental conditions; possible metabolism of ethylene by symbiotic bacteria; and possible toxicity of the reagents. ❐ *Porifera, nitrogen fixation, acetylene reduction activity, cyanobacteria, coral reef sponges, Callyspongia muricina.*

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Photosynthetic symbionts convey a distinct advantage to marine sponges living in low nutrient, tropical waters. These sponges have been shown to receive fixed nutrient carbon translocated from cyanobacterial symbionts (Wilkinson, 1979), such that some are virtually 'phototrophic', i.e. the symbionts can provide the bulk of carbon energy requirements (Wilkinson, 1983; Cheshire & Wilkinson, 1991). Such sponges are distinctly flattened to enhance light capturing, possibly at the expense of the ability to act as filter feeders (Wilkinson et al., 1988). These abilities are similar to other major photosynthetic symbioses on coral reefs: zooxanthellate corals (Muscatine et al., 1981); tridacnid clams (Trench, 1987); and didemnid ascidians (Griffiths & Thinh, 1983).

The potential to fix nitrogen was demonstrated in Red Sea sponges using the acetylene reduction test (Wilkinson & Fay, 1979). However, subsequent attempts to apply similar methods to a range of sponges from other coral reef areas proved to be highly variable. After many experiments showing equivocal results in 23 species, more direct and unambiguous nitrogen fixation methods were applied with the incubation of a sponge in the stable isotope $^{15}N_2$. We chose a sponge from the Great Barrier Reef that had previously shown some promise at fixing nitrogen and from which cyanobacterial cell preparations could be easily obtained.

Marine sponges also have many other photosynthetic and non-photosynthetic symbionts (Wilkinson, 1992). Many sponges have large populations of symbiotic bacteria, which may occupy up to 40% of the animal volume, comparable to the volume of the matrix and exceeding the volume of animal cells (Vacelet & Donadey, 1977; Wilkinson, 1978). These symbionts are particularly variable with the possibility of six or more 'species' occurring within the mesohyl matrix. However, few, if any, roles have been demonstrated for these symbionts, until recent experiments demonstrated a possible role in nitrogen metabolism within the sponge that also may be significant within coral reef ecosystems (Corredor et al., 1988; Diaz & Ward, 1997). Recently, many different bacterial forms have been observed, including Archaea-like cells (Preston et al., 1994; Fuerst et al., 1998) and methane-oxidising bacteria in sponges (Vacelet et al., 1996).

MATERIALS AND METHODS

All field experiments were performed at Davies Reef (18°15'S; 147°38'E) on the central part of the Great Barrier Reef aboard the RV Harry Messell (location in Wilkinson & Evans, 1989).

NITROGEN FIXATION BY ACETYLENE REDUCTION. The technique of Stewart et al. (1967) was used with incubations in 10-20% acetylene over acetylene saturated seawater (Flett et al., 1976). In some instances additional 5-20% oxygen gas was added to ensure that the sponges were not stressed through anaerobic conditions. Pieces (frequently transplanted several months prior to experimentation; Wilkinson & Thompson, 1997) or whole sponges of 23 species (116 replicates) were incubated in air tight containers, temperature buffered in running seawater and illuminated by filtered sunlight. Controls were either sponge tissue incubated in the dark, or boiled sponge tissue, or live coral rubble incubated in the light. Cyanobacterial cell preparations (see below) were incubated similarly.

Regular gas samples were collected over 4-6 hour incubation periods in evacuated tubes and assayed in a Tracor 222 gas chromatograph, with concentrations determined against an internal methane standard with corrections for gas solubility (Wilkinson & Sammarco, 1983).

NITROGEN FIXATION WITH $^{15}N_2$. The sponge *Callyspongia muricina* was chosen because it had frequently shown some acetylene reduction activity and because large quantities of cyanobacteria can be extracted from sponge tissues relatively easily. Seawater was degassed in a stream of argon gas and then saturated overnight under a headspace of 4 parts $^{15}N_2$ (Amersham) to 1 part O_2 with stirring.

Cyanobacterial preparations were obtained by gently crushing pieces of sponge, and blending the suspension in a glass blender with a teflon piston. After repeated centrifugation, washing and resuspension, a pellet was obtained that was predominantly cyanobacteria (examined microscopically). Replicate whole pieces of sponge and cell suspensions were incubated in closed chambers with no gas spaces in $^{15}N_2$ seawater (as above) for 20, 40 and 60mins.

Approximately 3cm^2 of sponge tissue was fixed in ethanol: water: acetic acid (50:45:5) and then pulverised using a 2cm probe diameter polytron probe blender in a 50ml polyethylene centrifuge tube in 25ml of distilled water. The mixture was filtered through 'Miracloth' until the spicule mass was colourless with all cyanobacterial cells removed. The suspension was extracted through Dowex 50 and 1 ion exchange columns to yield amino acid fractions. Centrifuged pellets of the cyanobacterial suspensions were similarly fixed and then blended.

Each fraction was reduced to 2ml and Kjeldahl digested at 150°C for 1.5hrs, followed by 330°C for 3hrs to remove all water (Bergensen, 1980). Digests were immediately converted to ammonium sulphate salts to prevent ambient contamination (Volk & Jackson, 1979) by digesting in 0.1M H_2SO_4 followed by 10M NaOH to convert to an alkaline solution. After 4 days incubation at 50°C, digests were evaporated to dryness under argon and analysed. The fixed nitrogen in samples was driven off by mixing with NaOBr under an argon atmosphere, and the gas injected directly into VG 602D (Cheshire, UK) computerised mass spectrometer through an ethanol-dry ice moisture trap. Calibration was with CIG ultra high purity nitrogen calibrated against N-1 and N-2 international standards (IAEA, Vienna) and results expressed as delta notation on air nitrogen scale with a reproductibility of 20 replicate samples being less than 0.1ppm. Control samples of unlabelled sponge, cell suspensions and coral rubble were similarly assayed to detect natural levels of $^{15}N_2$.

SPONGE PARAMETERS. Wet weight, dry weight, surface area and chlorophyll a content (Jeffrey & Humphrey, 1975) were as outlined in Wilkinson (1983).

RESULTS

NITROGEN FIXATION BY ACETYLENE REDUCTION. Assays of 23 sponge species revealed either negative, ambivalent or slightly positive results for ethylene production (Table 1). Three species, *Callyspongia muricina*, *Ircinia ramosa* and *Collospongia auris* frequently showed slight, or on occasions significant positive acetylene reduction. However, there was little consistency or reproducibility of positive results in these species, with or without symbiotic cyanobacteria. Similar ambiguous results were obtained with incubations of 6 sponge species from coral reefs off the coast of Western Australia (Wilkinson, unpublished data).

Variations in oxygen or acetylene concentrations on incubations of whole tissue or cell preparations had no apparent effect on acetylene reduction rates. Control incubations of coral

TABLE 1. Results of acetylene reduction assays on coral reef sponges, compared to pieces of rubble as positive controls. Negative controls of incubations in the dark or with dead sponge are not reported as all were negative. Results from experiments using different concentrations of oxygen and or acetylene are combined, as there were no discernible patterns. Symbiont = lists of the type of cyanobacterial symbiont. The Results are reported as the number of sponge incubations in each category: – = no evolution of ethylene, and less than contamination; +/– = inconclusive result with traces of ethylene; + = slight release of ethylene but less than 0.5 nM cm^{-2} hr^{-1}; ++ = significant increase in ethylene >2.0 nM cm^{-2} hr^{-1}. Nutrition = whether or not sponge can obtain the bulk of their carbon energy via symbiont photosynthesis (phototrophic), versus none (heterotrophic), or both (mixed).

Order	Species	Symbiont	Nutrition	–	+/–	1	1
Dictyoceratida	*Ircinia ramosa*	Unicellular	Phototrophic	1	[illegible]	[illegible]	0
	Phyllospongia lamellosa	Unicellular	Phototrophic	0	1	0	0
	Phyllospongia papyracea	Unicellular	Phototrophic	0	2	1	0
	Carteriospongia foliascens	Unicellular	Phototrophic	1	[illegible]	1	0
	Carteriospongia flabellifera	Unicellular	Phototrophic	2	2	0	0
	Strepsichordaia lendenfeldi	Unicellular	Mixed	[illegible]	[illegible]	0	0
	Collospongia auris	Unicellular	Phototrophic	1	1	2	0
	Ircinia sp. 1	Multicellular	Mixed	0	2	0	0
	Rhopaloeides odorabile	None	Heterotrophic	0	2	0	0
Dendroceratida	*Dysidea herbacea*	Multicellular	Phototrophic	0	[illegible]	0	0
	Dysidea sp. 1	Multicellular	Phototrophic	0	1	0	0
Haplosclerida	*Callyspongia muricina*	Unicellular	Phototrophic	7	23	[illegible]	0
	Callyspongia sp. 1	None	Heterotrophic	0	[illegible]	0	0
	Amphimedon sp. 1	Unicellular	Phototrophic	0	2	0	0
Petrosida	*Xestospongia exigua*	Unicellular	Mixed	0	2	[illegible]	0
Axinellida/ Halichondrida	*Cymbastela* sp. 1	Uni & Multi	Phototrophic	7	20	1	[illegible]
	Cymbastela sp. 2	Uni & Multi	Phototrophic	0	2	0	0
	Phakellia aruensis	None	Heterotrophic	0	1	0	0
	Acanthella sp.1	None	Heterotrophic	0	2	0	0
Poecilosclerida	*Neofibularia irata*	Unicellular	Phototrophic	0	3	0	0
Astrophorida	*Jaspis stellifera*	Unicellular	Mixed	0	2	0	0
Hadromerida	*Cliona* sp. BP	Zooxanthellae	Phototrophic	0	1	0	0
Class Calcarea	*Pericharax heteroraphis*	Unicellular	Mixed	0	1	0	0
None	Coral rubble controls	Turf algae		0	0	0	8

rubble with natural turf algal populations showed consistent, relatively high rates of acetylene reduction, comparable to those shown by Wilkinson et al. (1984).

NITROGEN FIXATION WITH $^{15}N_2$. Definite nitrogen fixation of $^{15}N_2$ was observed in whole sponge and cyanobacterial cell preparations of *C. muricina*. The highest rates of enrichment were observed in the amino acids: glutamine, glutamate and aspartate (Fig. 1). Similar rates of enrichment in the amino acid fraction were found in pieces of rubble incubated in $^{15}N_2$.

CELLULAR NATURE OF THE SYMBIONTS. The nature and location of cyanobacterial symbionts varies between sponges (Table 1). These were observed during transmission electron microscopic study of these sponges for other studies (Wilkinson, unpublished data). Three distinct categories can be observed: a) cyanobacteria free living in the mesohyl; b) cyanobacteria predominantly within large vacuoles within special mesohyl cells, cyanocytes; or b) and c) cyanobacteria both within vacuoles and in the mesohyl (Fig. 2; Wilkinson, 1978). In addition, some sponge species have other symbionts including multicullular cyanobacteria and zooxanthellae (Table 1; Wilkinson, 1992).

DISCUSSION

Two conclusions are evident from these studies: 1) at least one sponge with cyanobacterial

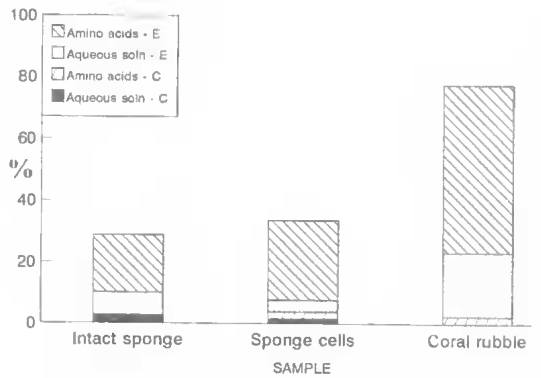

FIG. 1. Total enrichment of $^{15}N_2$ in whole sponge and cell suspensions of *Callyspongia muricina* compared to coral rubble controls. Data are delta enrichment values of $^{15}N_2$ compared to non-labelled N within control (C) aqueous and amino acid solutions and experimental (E) aqueous and amino acid fractions. Differences between E and C are significant at $P<0.001$.

symbionts fixes atmospheric nitrogen, but not at rapid rates, as indicated by direct fixation of $^{15}N_2$; and 2) the relatively easier technique of acetylene reduction is unreliable and inapplicable. A presumptive conclusion is that nitrogen fixation may occur in many other sponges with symbiotic cyanobacteria, but this can only be verified with individual testing of direct incorporation of $^{15}N_2$.

The first conclusion confirms the earlier observations of acetylene reduction in two Red Sea sponge species by Wilkinson & Fay (1979). We subsequently questioned those earlier results when repeated acetylene reduction tests on a larger number of species showed inconsistent results (Table 1). However, the direct incorporation of $^{15}N_2$ as gas has demonstrated that sponges with cyanobacterial (or possibly other prokaryotic) symbionts do contain active nitrogenase.

The progressive enrichment of glutamine, glutamate and aspartate demonstrate that this fixed nitrogen is of potential benefit to the host sponge as these compounds can be incorporated into sponge and symbiont protein, and metabolised for energy. Translocation of these amino acids, however, was not demonstrated, but may be assumed because the population size of microbial symbionts is usually stable with little need for protein for cell growth and there is a parallel release of fixed carbon as glycerol (Wilkinson, 1979).

Any nitrogen fixation would be valuable to those coral reef sponges that live in clear tropical waters, as it can supplement the particularly low levels of particulate nutrients and dissolved fixed nitrogen (Wiebe et al., 1975). Moreover, many of these sponges obtain the bulk of their energy from the photosynthetic symbionts as translocated glycerol, which is rich in carbon but devoid of nitrogen (Wilkinson, 1979, 1983). Without this added source of nitrogen, sponge growth rates could be reduced through a lack of nitrogen to produce proteins, particularly for the production of the fibrous protein skeleton. The majority of sponges in clean water on the Great Barrier Reef are distinctly flattened to enhance light capture (Wilkinson, 1988). These are sponges that exhibit particularly phototrophic nutrition and have the potential to obtain virtually all of their nutrition from the symbionts down to a depth of 30m (Cheshire & Wilkinson, 1991).

The following possible explanations are advanced to explain the inconsistency in acetylene reduction assays compared to coral rubble controls: 1) poor diffusion between multiple layers of cell and matrix; 2) disturbance to host sponges; 3) possible removal of ethylene by symbiotic bacteria; and 4) possible acetylene toxicity to sponges and cells.

1) The turf algae on the rubble are totally exposed to the seawater containing dissolved acetylene, with only the algal cell barriers remaining for ethylene to diffuse back into the water. Therefore, there is efficient and rapid transfer of both the acetylene into turf cyanobacteria and similar transfer of the ethylene back into seawater, evident as high and consistent rates of ethylene production (Table 1).

The situation in sponges is different as the symbionts in many sponges are contained within specialised vacuoles (cyanocytes; Wilkinson, 1978) embedded in the sponge mesohyl matrix (Fig. 2). For these symbionts, there are multiple cell and matrix layers that must be passed for both acetylene to diffuse into the cyanobacteria (or bacterial symbionts) and then for the ethylene to diffuse back out to the water, where it can be detected in water samples. This double diffusion process may be slow and inefficient, as it would rely on diffusion gradients across cell and matrix barriers.

Against this argument is the fact that low molecular weight gaseous molecules like acetylene (M.W. 26) and ethylene (M.W. 28) diffuse rapidly through membranes, comparable to other gases such as nitrogen (N_2 and $^{15}N_2$; M.W. 28 and 30) and oxygen (M.W. 32) (Cheung & Marshall, 1997).

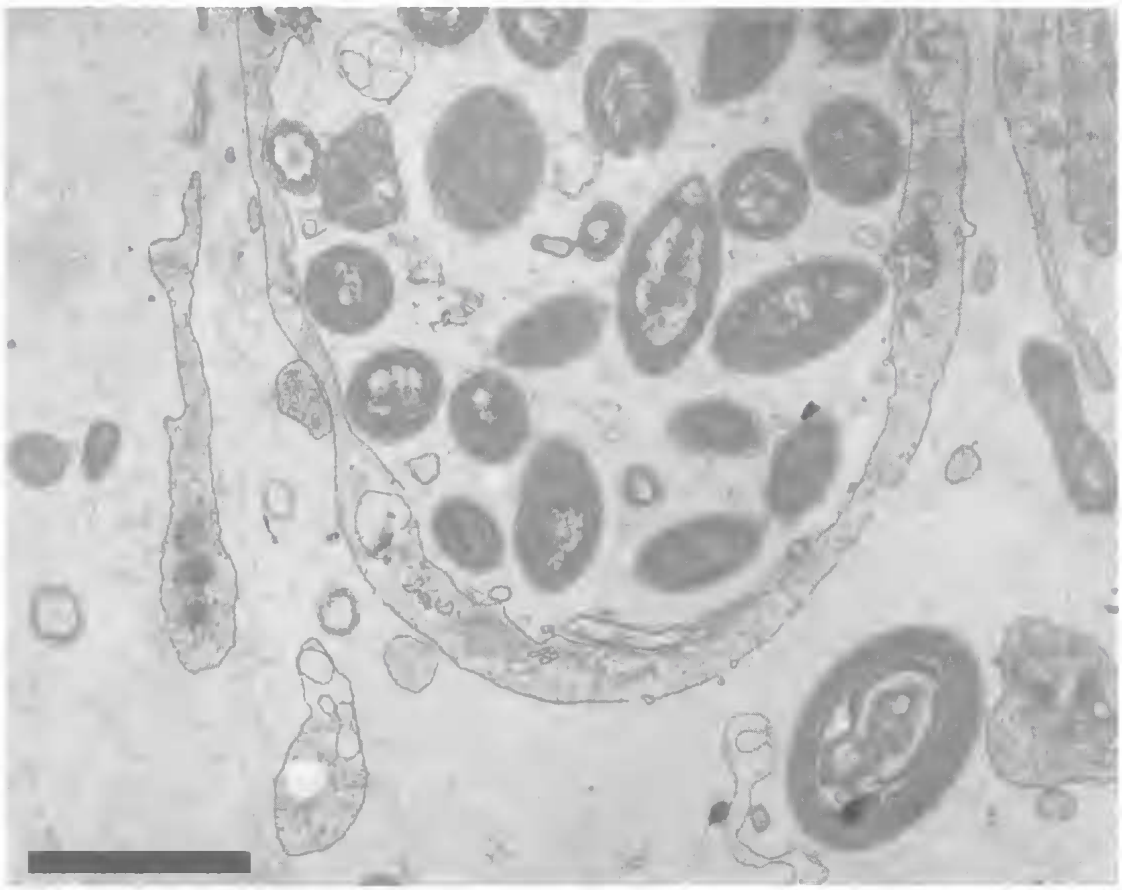

FIG. 2. Electron micrograph of the Great Barrier Reef sponge *Jaspis stellifera* showing unicellular cyanobacterial symbionts both free in the mesohyl matrix and contained within vacuoles of specialised cells, the cyanocytes. Scale bar 5μm.

2) Sponges have the ability to contract and cease pumping when disturbed, which would reduce water and gaseous exchanges. This was demonstrated by Reiswig (1971) and has been a consistent problem with physiological experiments with sponges on the Great Barrier Reef, because they frequently contract and close their oscules when placed in experimental chambers. This is most evident in massive species with large oscules, like *Rhopaloiedes odorabile* and *Jaspis stellifera*, but may also occur in small oscule species like the *Phyllospongia* and *Carteriospongia* spp., but observing any contraction in the field is particularly difficult. Cessation of pumping activity would restrict water movement through canals and prevent a free exchange of acetylene and ethylene, thereby reducing the potential for acetylene reduction.

3) There is the possibility that symbiotic bacteria exist which can oxidise either or both acetylene and ethylene and interfere with concentration measurements. Sponges contain a large variety of bacterial symbionts (Vacelet & Donadey, 1977; Wilkinson, 1978) and recently it has been shown that there are methane-oxidising bacteria in marine sponges (Vacelet et al., 1996), as well as a wide range of Archaea-like bacteria (Preston et al., 1994; Fuerst et al., 1998). The majority of bacterial symbionts cannot be isolated in culture and have only been observed using electron microscopy.

4) Acetylene toxicity has not been shown in these sponges, but has been demonstrated in other nitrogen fixing systems (David & Fay, 1977). Most acetylene reduction assays have been applied to plants, rather than animals. Thus acetylene toxicity may have a greater impact on

animal respiration and reduce or block the transfer of water through the canal system. This would result in the effects in 2) above.

Irrespective of the reason for inconsistencies with the acetylene reduction method compared to the use of the stable nitrogen isotope technique, it is concluded that acetylene reduction should not be used with these animals as a method to detect nitrogen fixation. One problem is that stable isotope analysis is more expensive and difficult to apply under field conditions. However, the ready availability of new continuous-flow isotope analyses methods for carbon, nitrogen and hydrogen isotopes means that enrichment experiments are very easily evaluated at the molecular level using compound-specific isotope analyses (Merrit & Hayes, 1994; Macko et al., 1997).

Verified nitrogen fixation in one sponge species has demonstrated the potential for fixation to be found in other sponges with microbial symbionts. Although it is more probable that the cyanobacterial symbionts are responsible for the activity, the possibility exists that bacterial symbionts may also fix atmospheric nitrogen in other sponges. More research is needed to confirm the origin of the nitrogen fixing enzyme, nitrogenase. Irrespective of the source, any fixed nitrogen would supplement nutrition in coral reef sponges that must make a living in low nutrient tropical waters.

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